# Evidence for at Least Two Distinct Groups of Humoral Immune Reactions to Papillomavirus Antigens in Women with Squamous Intraepithelial Lesions[1]

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## Abstract

**Serological markers of squamous intraepithelial lesions (SILs), the precursors of cervical cancer, have not been studied extensively. To screen for antibody responses that might be associated with SILs, we measured IgG and/or IgA to nine antigens based on papillomaviruses, the infectious cause of SIL and cervical cancer, using an ELISA format. Cases were 59 women with low grade SIL (LSIL) and 38 with high grade SIL (HSIL). Controls were 50 women chosen to minimize the possibility that they ever had SILs [individuals who had no history of SIL and repeatedly tested negative for cervical human papillomavirus (HPV) DNA], frequency age-matched to cases. The data showed that five antibodies had strong positive associations with SILs and that one was inversely related to SILs. By studying these antibodies in pairs, furthermore, we found that case-control differences were enhanced. In particular, the combination of IgG to an epitope in the E6 protein of HPV 16 (E6:10) and IgA to HPV 16 virus-like particles (VLPs) was detected in 53% of LSILs and 65% of HSILs but only 9% of controls. These same responses were both negative in just 6% of LSILs and zero HSILs, compared to 59% of controls. Notably, E6:10 IgG and HPV 16 VLP IgA were not correlated with each other, and the other antibody responses positively associated with SILs could be broken into two groups: those correlated with E610 IgG and those correlated with HPV 16 VLP IgA. Overall, the data suggest that several papillomavirus antibodies may be strongly related to SILs, and that they can be divided into at least two independent groups of humoral immune reactions.**

## Introduction

Sensitive DNA hybridization methods detect HPVs[3] in most neoplastic lesions of the cervix (1–3). Although HPV serological methods are not as well developed, studies of HPV antibodies provide insights into the humoral immune responses to HPV and HPV-related lesions (4). Furthermore, characterization of serological assays that can diagnose HPV infection or detect HPV-associated neoplasia remains a major goal. Such antibody tests might be useful in seroepidemiological studies and, ultimately, in screening populations at risk of anogenital cancers (*e.g.*, as an adjunct to Pap smears) (5). Characterization of antibody responses that are protective against infection or disease is also important as it could help direct vaccine development efforts (6, 7).

Different papillomavirus antibodies and assays may provide different types of information and have different applications (8, 9). In this connection, exploratory studies are being conducted to determine associations between an assortment of papillomavirus antibodies and different stages in the natural history of HPV infection and neoplasia of the cervix (4, 8–13). In particular, little is known about humoral immune responses to cervical cancer precursors, called SILs. Few antibodies that can discriminate patients with SILs from normal controls have been identified. Most antibodies associated with cervical cancer in previous studies have failed to discriminate patients with SILs from normal controls (8, 10).

In this project, we screened promising papillomavirus antibodies for associations with SILs. The antibodies tested were chosen primarily because of their associations with anogenital cancers (4, 12–15). Only antibodies to VLPs containing the L1/L2 capsid proteins of HPV 16 (HPV 16 VLP) had been studied previously in SILs (11, 16). Cofactors such as age, smoking, and sexual history were examined to determine if they affected any of these relationships. In addition, to determine which humoral immune responses are linked and which are independent, all antibodies associated with SILs in this study were assessed for correlations with one another, and we determined whether by studying these antibodies in pairs, case-control differences could be enhanced.

## Patients and Methods

**Patients.** All patients were enrolled at Kaiser-Permanente medical clinics in Portland, Oregon between 1989 and 1994 as part of

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[3] The abbreviations used are: HPV, human papillomavirus; SIL, squamous intraepithelial lesion; LSIL, low-grade SIL; HSIL, high-grade SIL; VLP, virus-like particle; BPV, bovine papillomavirus.

a large natural history study of HPV infection and cervical neoplasia, involving over 24,000 women, described elsewhere (8). We studied 59 cases with LSIL, 38 cases with HSIL, and 50 controls. Cases were women with SILs diagnosed cytologically and who were still untreated at the time of blood collection. The selection of controls was more complex. Because this was an exploratory investigation, it was a priority to avoid bias to the null, which might have occurred if women with a history of the exposure being studied, SIL, were included in the comparison group. Therefore, controls were women chosen to minimize the possibility that they ever had cervical neoplasia, even if the lesions were extremely subtle and resolved. Specifically, we selected control women from among the 17,654 cytologically normal subjects in the natural history study, who had no history of SILs (by interview and review of medical records), who did not develop SILs during the period of observation (median, 14 months), and who were negative for the main cause of cervical neoplasia, HPV, each of two times tested (described below). From this subset of normal subjects, a group of individuals frequency age-matched to cases was selected as controls. The advantage of this disease-free control group, compared with others at low risk of SILs that might have been used (*e.g.*, virgins), was their similarity to cases; they were age-matched and attending the same clinics. A limitation, though, was that by using HPV DNA-negative subjects as controls, effects due to SILs *per se* could not be entirely distinguished from effects due to HPV, its main etiological agent.

**Clinical Methods and Interviews.** Pap smears were collected using an Ayre spatula and an endocervical brush. Exfoliated cervical cells for HPV DNA testing were collected just after pap smear using a cervicovaginal lavage with 10 ml of normal saline. Lavage specimens and serum were shipped on dry ice and stored at −70°C until tested.

For controls, Pap smears and HPV DNA tests were performed twice, at enrollment and at their selection as controls (when blood was drawn). For cases, the biological specimens tested in this investigation were collected after cytological diagnosis and prior to any treatment.

Structured interviews regarding risk factors for cervical neoplasia were also obtained from all patients. The interview requested information regarding a variety of possible risk factors for HPV and SILs. In advance of data analysis, we chose to investigate the relation of age, race, smoking, parity, and sexual history with HPV antibody results. These factors were chosen because there was reason to think that they might be associated with serological parameters (17, 18), as well as with detection of HPV and SILs (19).

**Laboratory Methods.** Laboratory personnel were masked to the identity of patients, and specimens were placed in random order to avoid batch affects. An ELISA format was used to measure antibody responses to a panel of synthetic papillomavirus peptides as well as to VLPs. In brief, intact HPV 16 VLPs were coated onto microtiter plates (Costar, Cambridge, MA) using cold PBS at concentrations to yield 50 ng/well and incubated overnight at 4°C. In the case of BPV VLPs, 250 ng/well was used. Disrupted HPV 16 and BPV VLPs were created by incubating the antigens in 0.1 M carbonate buffer (pH 9.6) for 2 h at room temperature prior to dilution and coating to plates. Synthetic peptides were coated to microtiter plates in 0.1 M Tris-HCl (pH 8.8) and incubated overnight. For the synthetic peptide ELISAs, plates were washed with PBS containing 0.05% Tween 20 (PBS-T). Sera were added at 1:30 dilution in 10% horse serum-PBS and incubated on the plates in duplicate for 2 h at 37°C. To detect serum IgG and IgA bound by antigen to each well, we added mouse monoclonal antibodies against human $\alpha$ or $\gamma$ chains (Eurodiagnostics, Apeldoorn, the Netherlands) and then $\gamma$ chain-specific peroxidase-conjugated goat antibodies against mouse IgG (Southern Biotechnology, Atlanta, GA). Finally, the ABTS peroxidase substrate (Boehringer) was added for 1 h. Differences in optical density were determined for each serum by subtracting the absorbances of wells coated only with buffer from those of wells coated with peptide. Each sample was tested in singlet, and sera that gave absorbances exceeding 0.3 on buffer-coated wells were excluded from the analysis. VLP assays were conducted similarly to the peptide ELISAs, except that incubations were conducted at room temperature, and for HPV 16 VLP IgG, the absorbances of wells coated with disrupted particles were subtracted from the reactivity to intact VLPs.

In controls, a PCR-based method was used to detect and type HPV DNA. Specifically, we used MY9/MY11 consensus primers, followed by probes able to identify HPV 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, and 68 and novel viruses PAP155, PAP238a, PAP291, and W13B, as well as a number of uncharacterized HPV types (20). This assay is sensitive to as few as 10 copies of viral DNA. Thus, the exclusion of HPV DNA-positive women from controls was based on highly sensitive methods (21).

In cases, Hybrid Capture (Digene Diagnostics, Silver Spring, MD) was used to detect cervical HPV, and we specifically probed for types 6, 11, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 56, and 58 (22). Although the use of sensitive PCR methods for detection of HPV in controls made it as certain as possible that these patients were indeed HPV DNA negative, the use of Hybrid Capture in cases restricted detection to moderate levels of HPV DNA expression known now to correlate with current SILs.

Cervical pathology was diagnosed by conventional cytology, reviewed for accuracy by a single consultant pathologist (D.R.S.).

**Statistical Analysis.** Exploratory data analysis showed that seroassay results, universally, were best normalized by log transformation. Generalized linear models were then used to examine the relation of log-transformed seroassay values with case-control status, and likewise, with other cofactors in univariable and multivariable analyses. Correlations between different antibodies were estimated using a Spearman correlation matrix. Categorical data were created from seroassay results by setting a cutoff in each assay, based on an *a priori* approach that proved useful in previous investigational studies (12, 14), *i.e.*, the mean of the geometric means of all groups (*i.e.*, controls, LSIL, and HSIL). Categorical data were analyzed by the $\chi^2$ test, the Mantel-Haenszel test for trend, or logistic regression.

To verify that the predefined cutoff points in each ELISA were representative, we compared our findings based on these categorical data to those determined using the continuous data, *i.e.*, the original (log-transformed) seroassay values. As a further step, two additional *a priori* cutoff definitions were also tested, each based on the distribution of seroassay values in controls: (*a*) above the third quartile of controls; and (*b*) one SD above the mean of controls. All methods of analysis gave similar results, including those using the latter two cutoff definitions (data not shown). Throughout this report, the categorical data refer to results based on the mean of the geometric means as the cutoff, and the exact cutoff levels used for each assay are reported in Table 1. The study was designed to detect only substantial differences between cases and controls.

*Table 1* Antibody prevalence in patients with LSILs and HSILs of the cervix and in HPV DNA-negative controls

| Antibody[a] | Controls (%)[b] | LSIL (%) | HSIL (%) |
|---|---|---|---|
| BPV IgA | 73 | 60[c] | 63[c] |
| BPV IgG | 83 | 40[d] | 57[e] |
| E1:18 IgA | 45 | 21[e] | 29 |
| E1:18 IgG | 48 | 64 | 81[e] |
| L1:13 IgA | 67 | 59 | 61 |
| L1:13 IgG | 53 | 42 | 71 |
| L1:31 IgG | 60 | 54 | 70 |
| E7:5 IgA | 45 | 59[c] | 66[f] |
| E2:9 IgA | 35 | 72[g] | 71[d] |
| E1:19 IgG | 37 | 67[e] | 78[d] |
| HPV16 VLP IgA | 27 | 71[g] | 74[g] |
| HPV16 VLP IgG | 28 | 62[d] | 74[g] |
| E6:10 IgA | 43 | 47 | 66[f] |
| E6:10 IgG | 28 | 76[g] | 92[g] |

[a] BPV, [disrupted VLPs derived from L1 open reading frame of BPV; IgA cutoff difference in optical density (dOD) = 51; IgG cutoff dOD = 358]; E1:18, [peptide antigen derived from middle region of E1 open reading frame (ORF) of HPV 16; IgA dOD = 3.5; IgG dOD = 42]; L1:13, peptide antigen derived from the middle part of the L1 ORF of HPV 16; IgA dOD = 73; IgG dOD = 262); L1:31, peptide antigen derived from COOH terminus of the L1 ORF of HPV 16; IgG dOD = 77; E7:5, peptide antigen derived from E7 ORF of HPV 16; IgA dOD = 74; E2:9, peptide antigen derived from middle part of E2 ORF of HPV 16; IgA dOD = 13; E1:19, peptide antigen derived from middle region of E1 ORF of HPV 16; IgG dOD = 32; HPV 16 VLP, VLPs derived from L1 ORF of HPV 16; IgA dOD = 21; IgG dOD = 98; E6:10, peptide antigen derived from COOH terminus of E6 ORF of HPV 16; IgA dOD = 9.5; IgG dOD = 47.

[b] Data for each antibody response was obtained from never less than 46 of the 50 controls, never less than 50 of the 59 LSILs, and never less than 37 of the 38 HSILs, after excluding subjects for unavailability of remaining sera or absorbances of greater than 0.3 in buffer-coated wells run for each subject with each assay.

[c] Based on data analyzed as continuous values, but not on prevalence rates, results were significantly different than in controls ($P < 0.05$).

[d] Prevalence rate is significantly different than in controls ($P < 0.05$).

[e] Prevalence rate is significantly different than in controls ($P < 0.01$).

[f] Prevalence rate is significantly different than in controls ($P < 0.001$).

[g] Prevalence rate is significantly different than in controls ($P < 0.0001$).

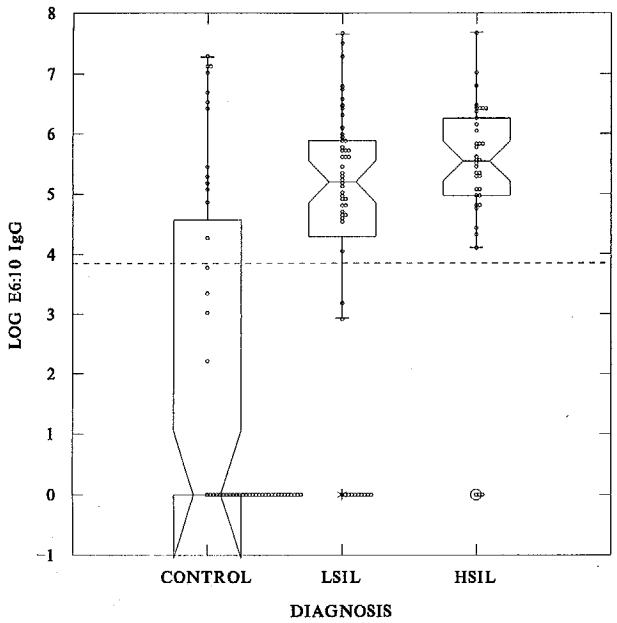


*Fig. 1.* IgG to E610 in subjects with LSILs or HSILs and controls. The values shown are absorbance results in the ELISAs after subtracting background and log transformation. Only zero values were not log transformed. *Boxplots* are interpreted as follows. The *waist* is the median, *diagonal lines* indicate 95% confidence intervals about the median; *lower* and *upper horizontal lines* indicate 25th and 75th percentiles of the distribution; *upper* and *lower bars* (*fences*) indicate the range of the data excluding outliers. *Points* marked with *asterisks* represent outliers [*i.e.,* values beyond the fences: 25th percentile − (1.5 × interquartile range); 75th percentile + (1.5 × interquartile range)]; *points* marked with *circles* represent far outliers [*i.e.,* 25th percentile − (3 × interquartile range); 75th percentile + (3 × interquartile range)]; and the *dotted horizontal line* across the figure shows the cutoff value in the assay, which was 3.85.

## Results

**General.** Age was not different between cases and controls, suggesting that this factor was well controlled for by frequency age-matching. Race was also similar in cases and controls, as essentially all patients were Caucasian. Cases, though, did differ as expected from controls, in terms of smoking and sexual experience. However, after accounting for case-control status in multivariable logistic regression, these behavioral factors did not affect the antibody responses measured (data not shown).

**Antibody Responses in Cases and Controls.** Antibody responses to papillomavirus antigens were compared between cases and controls. Table 1 shows seroprevalence rates in all of the subjects tested. The results were almost always similar to findings based on the actual seroassay values analyzed as continuous variables. Indeed, the direction of case-control differences was always the same, and Table 1 indicates the few examples for which statistical significance was different in the two sets of analyses.

Case-control differences were found for several HPV antibodies. Most of these antibodies were more prevalent in cases (described more fully below). However, as in previous studies, antibody responses that were less common in patients with SILs were also observed (8, 12, 14). The strongest of these inverse associations was for IgG to BPV (disrupted virus-like particles of BPV type 1), which was positive in 83% of controls but only 40% of LSILs and 57% of HSILs. Likewise, IgA to BPV and IgA to E1:18 (an antigen derived from the middle region of the E1 protein of HPV 16) were more common in controls than cases.

In contrast, IgG to E6:10 (a peptide from the E6 COOH terminus of HPV 16) was significantly and substantially more prevalent in cases than controls (Fig. 1). Specifically, among cases, 76% with LSILs and 92% with HSILs had IgG to E6:10, as compared to only 28% of controls.

IgG and IgA to HPV 16 VLP, VLPs containing the L1/L2 proteins of HPV 16, were also considerably more prevalent in patients with SILs, and as with E6:10 IgG, prevalence increased with increased grade of neoplasia. IgA to HPV 16 VLP was positive in 71% of LSILs and 74% of HSILs as compared to 27% of controls. IgG to HPV 16 VLP was positive in 62% of LSILs and 74% of HSILs, as compared to 28% of controls.

Two other antibody responses were significantly more common in patients with both LSILs and HSILs. IgG to E1:19 (a peptide derived from the middle region of E1 of HPV 16 and overlapping with E1:18) and IgA to E2:9 (an antigen made from the middle part of E2 of HPV 16) were positive, respectively, in 67 and 72% of patients who had LSILs and in 78 and 71% of patients who had HSILs but only 37 and 35% of controls. The only other antibody response strongly associated with SILs was IgG to E1:18, but this relationship was primarily with HSILs. Specifically, 81% of HSIL cases was positive for E1:18 IgG as compared to 64% of LSILs and 48% of controls.

**Correlations between Selected Antibody Responses.** Based on their mutually strong association with SILs, IgG to E6:10 and IgA to HPV 16 VLP might have been expected to be highly

*Table 2* Selected HPV antibody responses considered in combination: prevalence rates in cases with LSILs or HSILs (as a single group) and in controls

| Antibody | | E610 IgG (%) | | | HPV 16 VLP IgA (%) | | |
|---|---|---|---|---|---|---|---|
| | | Both positive | Either positive[a] | Both negative | Both positive | Either positive | Both negative |
| E610 IgG | Cases[b] | | | | 57 | 95 | 5 |
| | Controls | | | | 9 | 47 | 53 |
| HPV16 VLP IgA | Cases | 56 | 97 | 3 | | | |
| | Controls | 9 | 41 | 59 | | | |
| HPV16 VLP IgG | Cases | 57 | 95 | 5 | 52 | 86 | 14 |
| | Controls | 9 | 47 | 53 | 8 | 45 | 55 |
| E1:19 IgG | Cases | 71 | 83 | 17 | 50 | 93 | 7 |
| | Controls | 26 | 39 | 61 | 9 | 51 | 49 |
| E29 IgA | Cases | 56 | 98 | 2 | 60 | 82 | 18 |
| | Controls | 7 | 54 | 46 | 8 | 47 | 53 |

[a] Either or both of the two antibodies positive.
[b] All case control comparisons were highly significant ($P < 0.001$).

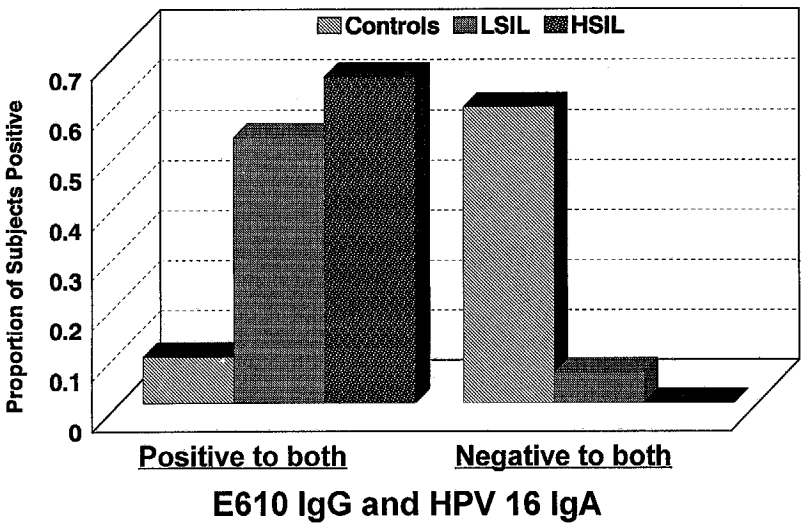


*Fig. 2.* Concordance of antibodies in cases and controls.

correlated. However, their Spearman rank correlation was only 0.13 ($P = 0.13$), suggesting that they might measure different aspects of the immune response to SILs. In fact, the humoral immune response to SILs in general might be split into two or more distinguishable components. That is, antibodies to E1:19 and E6:10 (IgG and IgA) were correlated, and antibodies to E2:9 and HPV 16 VLP (IgG and IgA) were correlated, but the two sets of antibodies were not associated with each other.

Specifically, IgG to E1:19 was strongly associated with IgG to E6:10 (Spearman $r = 0.87$; $P < 0.0001$) and moderately with IgA to E6:10 (Spearman $r = 0.26$; $P = 0.002$) but not with either IgG to HPV 16 VLP (Spearman $r = 0.03$; $P = 0.75$), IgA to HPV 16 VLP (Spearman $r = 0.06$; $P = 0.49$), or with IgA to E2:9 (Spearman $r = 0.11$; $P = 0.22$). The reverse pattern was then observed for IgA to E2:9. E2:9 IgA was associated with IgA to HPV 16 VLP (Spearman $r = 0.42$; $P < 0.0001$) and with IgG to HPV 16 VLP (Spearman $r = 0.29$; $P = 0.0005$) but not either IgG to E6:10 (Spearman $r = 0.06$; $P = 0.49$) or IgA to E6:10 (Spearman $r = 0.08$; $P = 0.33$). In addition, IgG and IgA responses to a single given HPV epitope were related, but to varying extents. IgG and IgA to HPV 16 VLP were strongly correlated with each other (Spearman $r = 0.46$; $P < 0.0001$), and IgA and IgG to E6:10 were more weakly correlated with each other (Spearman $r = 0.20$; $P = 0.02$).

Finally, given the inverse association of IgG to BPV with SILs, an inverse association with the above antibody responses might have been expected. However, only IgA to HPV 16 VLP (Spearman $r = -0.23$; $P = 0.008$) showed even a moderate positive or negative association with IgG to BPV.

**Positive Results in More than One Seroassay.** We considered in pairs the results of the SIL-related antibody responses to measure the association of their combined results with SILs. For example, E6:10 IgG and HPV 16 VLP IgA were each individually and strongly associated with SILs, as above. However, the relation with SILs was much stronger still if both antibodies were concordantly positive (Table 2, which combines data from LSILs and HSILs, and Fig. 2). Specifically, 53% of LSILs and 65% of HSILs were positive for both E6:10 IgG and HPV 16 VLP IgA, as compared to just 9% of controls. Furthermore, just 6% of LSILs and no HSILs at all were concordantly negative for both antibodies, whereas 59% of controls were negative for both.

Therefore, as expected, direct positive associations between antibody responses and SILs were even stronger when considered in combination. In addition, the magnitude of this effect (Table 2) was generally related to the strength of association of each of the individual antibody responses with SILs (Table 1), with one notable exception. Concordant positive results for E6:10 IgG and E2:9 IgA showed an even stronger

relation to SILs than anticipated. In 62% of HSILs and 51% of LSILs, both antibodies were positive, as compared to only 7% of controls. In addition, antibodies to E6:10 IgG and E2:9 IgA were never concordantly negative in HSIL, and both antibody responses were absent in just 4% of LSIL. In contrast, 46% of controls lacked antibodies to both E6:10 IgG and E2:9 IgA.

BPV IgG, which was inversely associated with SIL, was also studied in combination with E6:10 IgG, with HPV 16 VLP IgG, and with HPV 16 VLP IgA (all direct positive associations with SILs). The expectation was that the strongest inverse associations would be observed when IgG to BPV was positive and a paired (positively associated) antibody was negative. Indeed, only 3% of HSILs and 4% of LSILs were positive for IgG to BPV and negative for IgG to E6:10, as compared to 62% of controls. Likewise, similar patterns were observed for BPV in combination with HPV 16 VLP IgG and with HPV 16 VLP IgA.

**HPV DNA and Papillomavirus Antibodies.** All controls, by design, were HPV DNA negative by PCR at the time of blood draw and in previous testing at entry into the overall natural history study. In contrast, cases commonly had HPV DNA detected in their cervical specimens by the Hybrid Capture test. The detection rate for HPV DNA in HSILs (67%) and LSILs (56%), however, was somewhat lower than expected, and the detection rate for HPV 16 DNA, which is typically much greater in severe cervical neoplasia, was similar ($P = 0.39$) in patients who had HSILs (47%) and LSILs (38%). In effect, only the HPV DNA-positive cases were entirely interpretable. Therefore, we restricted our analyses to the examination of whether HPV 16 DNA detection in cases affected HPV 16 antibody responses. The data showed that antibody to HPV 16 VLPs, but not other antigens, had limited specificity for the detection of HPV 16 in the cervix. Specifically, HPV 16 VLP IgG was detected in 74% of HPV 16 DNA-positive cases ($n = 38$) as compared to 44% of cases ($n = 18$) with other HPV DNA types, a significant difference even after adjusting for grade of lesion ($P = 0.04$). Likewise, HPV 16 VLP IgA was detected in 79% of HPV 16 DNA but only 61% of cases with other HPV DNA types, albeit this difference was not statistically significant ($P = 0.13$).

## Discussion

Characterization of HPV antibody responses that are strongly associated with the detection of cervical cancer precursors has obvious clinical and investigational utility. Previously, however, few antibodies associated with SILs had been identified (4, 8, 9). Even antibodies linked to cervical cancer had generally failed to distinguish patients with SILs from normal controls, in earlier investigations (8, 10). In this study, though, we identified several antibody responses that were strongly associated with the diagnosis of current LSILs and HSILs.

IgG to E6:10, a peptide derived from the COOH terminus of the E6 molecule of HPV 16, was the antibody most strongly associated with SILs in this study. Almost all patients who had HSILs and three quarters of those who had LSILs were found to have IgG to E6:10, as compared to only one-third of controls. We did not measure sensitivity or specificity of the E6:10 IgG assay for SILs, because this was not a population-based trial. For example, seroprevalence among controls may have underestimated HPV antibody prevalence in the broader community of all women who never developed SILs. However, the importance of the strength of the E6:10-SIL association is highlighted by the observation that several other cancer-associated antibodies (*e.g.*, L1:13 IgG and E1:18 IgA) were not associated with SILs in this study, as well as the difficulty previous investigations had in finding any antibody responses that even weakly discriminated patients with SIL from normal controls (4, 8, 10). E6 is one of two HPV proteins, along with E7, that can immortalize keratinocytes *in vitro* and, like E7, it is thought to have an important role in the etiology of cervical neoplasia (23, 24). Several papers have reported that antibodies to E6 or E7 antigens are associated with cancer (10, 25–27). However, E6 expression in SILs is probably much less than in cancer (23, 24). Thus, it is interesting that we not only detected an association of antibody to E6 with SIL but that the relationship was strong, even for LSIL.

We reported recently that both IgA and IgG to E6:10 were associated with invasive cervical cancer (12). In the present study, however, IgG but not IgA to E6:10 had a strong association with SIL. The reasons for this discrepancy must be investigated further in future studies. There could be immunoglobulin class-specific differences in the immune responses to invasive cancer and SILs, as we have suggested previously (8).

IgG and IgA to HPV 16 VLP, VLPs containing the L1/L2 capsid proteins of HPV 16, were also strongly associated with SILs. HPV 16 VLP IgG has been characterized previously in patients with SIL, and the high prevalence in patients with both LSILs and HSILs in this study was consistent with those reported in earlier investigations (11, 16). Strong relationships with SILs were also found for E1:19 IgG and E2:9 IgA, peptides derived from the E1 and E2 regions of HPV 16, respectively.

That the five antibody responses positively related to SILs were not universally correlated with each other was intriguing. The correlation matrix suggested that there are at least two separate groups of humoral immune responses to SILs. Specifically, the first group includes IgG to E6:10 and IgG to E1:19. The second group includes IgG and IgA to HPV 16 VLP and IgA to E2:9. These findings concur with results obtained by our laboratory in a recent study of patients with frank carcinoma of the cervix (12). It is not clear, however, why certain immunoglobulin responses to HPV antigens are correlated and others are not. Specifically, it remains uncertain what antigen characteristics predict which particular antibodies (against even dissimilar epitopes) are related. HLA type and other genetic factors, though, probably play important roles in determining the HPV epitopes against which an individual will mount humoral immune responses. If it is confirmed by others that papillomavirus antibodies in women with cervical neoplasia can be divided into two or more distinct and independent groups of humoral immune reactions, it may point to an important immunological phenomenon.

Considered in pairs, the five antibody responses positively associated with SILs showed greatly enhanced associations with cervical neoplasia. For example, almost two-thirds of patients who had HSILs and about one-half with LSILs were concordantly positive for E6:10 IgG and HPV 16 VLP IgA as compared to 9% of controls. Furthermore, no women with HSILs and just 6% of those with LSILs were concordantly negative, compared to 59% of controls. Similar results were also found for the other SIL-related antibodies in combination. In view of recent suggestions that serology could be used as an adjunct to Pap smears (5), these findings are intriguing and suggest that a panel of antibody assays, rather than a single test, might eventually turn out to be the better approach to serological screening for cervical neoplasia.

In contrast with the above, IgG to disrupted BPV capsids, a group-specific antigen, was inversely associated with cervical neoplasia, consistent with previous reports in cancer patients (12, 14). However, the question remains whether IgG to this group-specific antigen directly protects against development of

cervical neoplasia. Because BPV IgG was most common in controls, individuals who had the least extensive sexual experience, it is possible that this is a cross-reactive antibody response reflecting past exposure to infection with nonsexually transmitted and possibly non-anogenital (*e.g.,* cutaneous) HPV. If such an antibody were indeed cross-reactive against sexually transmitted anogenital HPV, it could conceivably lead to a protective effect. More likely perhaps, BPV IgG could just be a marker of another yet undefined factor inversely associated with cervical neoplasia.

Several risk factors for cervical neoplasia, including, smoking, sexual behavior, age, and race were examined to determine if they affected the relationship of antibody responses with SILs. However, none of these factors significantly influenced results. Thus, for this data set, differences in antibody levels were adequately explained by case-control status, with one important exception. HPV 16 VLP levels were slightly and positively affected by infection with HPV 16. None of the other ELISAs tested, though, showed any HPV 16 DNA specificity at all.

These observations are consistent with earlier reports that suggested that antibodies to intact HPV 16 VLPs are induced by HPV infection *per se* and are partially HPV 16 specific (11). Previous studies also suggested that, in contrast to VLP antibodies, antibodies to linear HPV peptides are not HPV type specific and might only be detected after the development of cervical neoplasia (*e.g.,* after prolonged, increased expression of specific HPV antigens; Refs. 8 and 9). In the present exploratory study, we could not analytically separate the effects of HPV infection *per se* from those of cervical neoplasia. However, the lack of HPV 16 type specificity in antibody responses to linear HPV peptides was as expected, and it is most likely that (as with other linear HPV peptide antibodies) serological responses to linear HPV peptides among cases and controls in this study are accounted for by cervical neoplasia and not HPV infection alone. To specifically address these issues, though, will require a separate investigation.

Papillomavirus seroepidemiology is a growing area of research that has been primarily limited by the unavailability of well-characterized seroassays. In this report, we described several papillomavirus antibodies that have strong positive associations with current SILs, especially when used in pairs, and one that is inversely associated with SILs. The data also suggested that these antibodies could be divided into at least two distinct and independent groups of humoral immune reactions based on their correlations with one another. The most discriminating of the papillomavirus antibodies should now be studied in population-based seroepidemiological studies to further examine their sensitivity and specificity.

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